APPENDIX

Changes to Abstract:

The following is a marked-up version of the amended Abstract:

ABSTRACT OF THE DISCLOSURE

Posttranslational modification of histones, in particular acetylation and deacetylation are involved in the regulation of gene expression. Histone deacetylases remove acetyl groups from histone proteins. The present invention is directed to a method of regulating gene expression in a transgenic plant comprising, introducing into a plant a first chimeric nucleotide sequence comprising a first regulatory element in operative association with a ~~gene~~ coding seqeunce of interest, and an upstream activating sequence, and a second chimeric nucleotide sequence comprising a second regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase and a nucleotide sequence encoding a DNA binding protein, and growing the transgenic plant. Furthermore, a method for regulating gene expression of an endogenous ~~gene~~ coding seqeunce of interest, or modifying a developmental, physiological or biochemical pathway in a plant is provided comprising introducing into a plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase fused with a nucleotide sequence encoding a DNA binding protein capable of interacting with an endogenous controlling sequence, for example an upstream activating sequence, and growing the transgenic plant. This invention also relates to novel histone deacetylase obtained from plants, to novel chimeric construct comprising these, or other histone deacetylase, and to transgenic plants, plant cells, or seeds comprising these chimeric constructs.

Changes to Specification:

Page 3, lines 7-15:

According to the present invention there is provided a method of regulating gene expression in a transgenic plant comprising, introducing into a plant:

i) a first chimeric nucleotide sequence comprising a first regulatory element in operative association with a ~~gene~~ coding sequence of interest, and a controlling sequence~~;~~, and

ii) a second chimeric nucleotide sequence comprising a second regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase and a nucleotide sequence encoding a DNA binding protein, the DNA binding protein having an affinity for the controlling sequence,

to produce the transgenic plant, and growing the transgenic plant.

Page 7, lines 11-24:

FIGURE 2 shows nucleotide and predicted amino acid sequences of several more HD's of the present invention. Figure 2 (A) shows the nucleotide and amino acid of *AtHD2A* (SEQ ID NO's:5 and 6, respectively. Figure 2 (B) shows the nucleotide and amino acid of AtHD2B (SEQ ID NO's: 7 and 8, respectively).

FIGURE 3 displays the amino acid sequence alignment of the *AtRPD3A* (SEQ ID NO:2), *AtRPD3B* (SEQ ID NO:4), maize RPD3 (ZmRPD3) and yeast RPD3. Identical amino acids are shaded in black. The amino acids with asterisks represent residues with potential roles in deacetylase activity.

FIGURE 4 displays the amino acid sequence alignment of *AtHD2A* (SEQ ID NO:6), *AtHD2B* (SEQ ID NO:8) and maize HD2 (ZmHD2). Identical amino acids are shaded in black. The amino acids with asterisks are the predicted histone deacetylase catalytic residues. The extended acidic domains are underlined.

Page 10, line 19, to page 11, line 11:

FIGURE 17 shows an outline of an experiment to demonstrate repression of expression of a gene in a tissue-specific manner. Figure 17(A) outlines a binary transrepression system involving the use of a tissue-specific regulatory element and constructs shown in Figure 17 (B). A reporter gene (expression construct) under the control of a constitutive promoter is active when introduced into a reporter plant. Effector genes, under control of tissue specific regulatory regions are introduced into effector plants. Transgene repression is achieved by crossing reporter plant lines with effector lines that express a repressor (eg. histone deacetylase), and a controlling sequence binding domain that specifically recognizes a control sequences of the reporter gene. The pattern of reporter gene repression will reflect the pattern of repressor expression, allowing a ~~gene~~ coding sequence of ~~interested~~ interest to be repressed under a variety of regimes by crossing to an appropriate effector line. The upper lob of the schematic plant represents the fruiting body of the plant, for example the seeds, while the horizontal lobes represent leaves. Black areas represent tissues exhibiting reporter gene expression, while grey and white areas represent no reporter gene expression. Grey regions indicate expression of the HD/CS-BD (effector) constructs, for example, either NAP1-GAL4/HD, or tCUP-GAl4/HD. Figure 17 (B) shows a schematic of the plasmids used for repressing transgene expression in transgenic plants. The effector constructs contained the tCUP promoter (Effector 1) or napin promoter (NAP; Effector 2)) fused to the fusion of the GAL4BD with the *AtHD2A* coding region and the polyadenylation signal of nopaline synthetase gene (Nos-T). The reporter constructs ($GAL4_{UAS}$-tCUP-GUS and $GAL4_{UAS}$-35S-GUS) contained the upstream activating sequence of GAL4 protein tandem repeated two times ($GAL4_{UAS}$) fused to the -394-tCUP or 35S promoter-GUS constructs.

Page 14, lines 28-32:

As described in more detail below, a plant EST database was screened using yeast *RPD3* or maize *HD2*. Two EST clones were identified corresponding to the yeast *RPD3* sequence and two clones were identified corresponding to the maize *HD2* sequence (Figure 1 and 2). These clones were termed *AtRPD3A* (SEQ ID NO:1), *AtRPD3B* (SEQ ID NO:~~2~~ 3), *AtHD2A* (SEQ ID NO:~~3~~ 5) and *AtHD2B* (SEQ ID NO:4 7), respectively.

Page 15, lines 9-20:

The HD's of the present invention, and those of the prior art, may be used to repress the expression of a ~~gene~~ coding sequence of interest within a plant by targeting a desired HD to a nucleotide sequence containing the ~~gene~~ coding sequence of interest. While not wishing to be bound by theory, the repression of gene expression activity via locally altering chromatin structure is made possible by targeting a HD to a nucleotide sequence within the vicinity of a ~~gene~~ coding sequence of interest. The localized deacetylation of histones may result in the observed repression of transcription as described herein. By "histone deacetylase" (HD) it is meant any HD as known within the art. These include the HD's as described of the present invention as well as other plant, animal or microbial HD's. Furthermore, by "repression of gene expression activity" it is meant the reduction in the level of mRNA, protein, or both mRNA and protein, encoded by the ~~gene~~ coding sequence of interest. Repression of gene expression activity may result from the down regulation of transcription.

Page 18, lines 1-9:

The DNA sequences of the present invention include the DNA sequences of SEQ ID NO: 1, 3, 5 and 7 and fragments thereof, as well as analogues of, or nucleic acid sequences comprising ~~about substantial homology of~~ about 80% similarity with the nucleic acids as defined in SEQ ID NO's: 1, 3, 5 and 7. Analogues (as defined above), include those DNA

sequences which hybridize under stringent hybridization conditions (see Maniatis *et al.*, in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, p. 387-389) to any one of the DNA sequence of SEQ ID NO: 1, 3, 5 or 7 provided that said sequences maintain at least one property of the activity of the HD as defined herein.

Page 18, lines 23-26:

The present invention is further directed to one or more chimeric gene constructs comprising a ~~gene~~ coding sequence of interest operatively linked to a regulatory element. Any exogenous ~~gene~~ coding sequence can be used as a ~~gene~~ coding sequence of interest and manipulated according to the present invention to result in the regulated expression of the exogenous ~~gene~~ coding sequence.

Page 19, lines 27-30:

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. However, it is to be understood that the chimeric gene constructs of the present invention may also be combined with ~~gene~~ a coding sequence of interest for expression within a range of plant hosts.

Page 20, lines 1-15:

By "~~gene~~ coding sequence of interest" it is meant any ~~gene~~nucleotide sequence that encodes a protein and that is to be expressed within a host organism. Such a ~~gene~~ coding sequence of interest may include, but is not limited to, a ~~gene~~ coding sequence whose product has an effect on plant growth or yield, for example a plant growth regulator such as an auxin or cytokinin and their ~~analogous~~analogues, or a ~~gene~~ coding sequence of interest may comprise a herbicide or a pesticide resistance gene, which are well known within the art. A ~~gene~~ coding sequence of interest may also include a ~~gene~~ nucleotide sequence that encodes a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins

include, but are not limited to, interleukins, insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon-α, interferon-ß, interferon-τ, blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. A ~~gene~~ coding sequence of interest may also encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include, but are not limited to proteases, oxidases, phytases, chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis etc.

Page 21, lines 5-26:

The present invention relates to chimeric constructs and a method for regulating the expression of a ~~gene~~ coding sequence of interest through the use of at least one HD. The chimeric constructs include:

- a first chimeric construct (the expression construct) comprising a first regulatory element, a controlling sequence (CS), a ~~gene~~ coding sequence of interest, and a terminator. The first regulatory element may permit the constitutive, developmental or temporal expression of the ~~gene~~ coding sequence of interest within a plant; and
- a second chimeric construct (the effector construct), comprising a second regulatory element, a ~~gene~~ nucleotide sequence encoding a CS binding domain (CS-BD), and HD, and a terminator sequence. The second regulatory element may permit the constitutive, developmental, temporal or induced expression of the HD within a plant.

The method includes introducing the first and second chimeric constructs as described above, within a plant in order to obtain controlled expression of the ~~gene~~ coding sequence of interest. The introduction of the two chimeric constructs within a plant may take place using techniques well known within the art such as transformation wherein both chimeric

constructs are introduced into the same plant, or through mating plants that each comprise one of the desired constructs in order to obtain a plant that expresses both chimeric constructs.

Page 21, line 28, to page 22, line 3:

The CS and CS-BD are characterized in that they exhibit an affinity for each other and are capable of interacting *in vivo*. In this manner, the product of the effector construct, comprising CS-BD and HD, is targeted to the CS of the expression construct. Results described herein demonstrate that the activity of the expression construct is repressed through the targeting of an effector construct product comprising HD. While not wishing to be bound by theory, this repression may result from the localized deacetylation of histones by HD which results in the repression of transcription of the ~~gene~~ coding sequence of interest.

Page 22, lines 5-28:

By "controlling sequence" (or CS) it is meant, a nucleotide sequence, for example, but not limited to, a regulatory region of a gene, that interacts with a DNA binding protein. However, a CS may include any nucleotide sequence that interacts with a DNA binding protein. The CS is preferably located in proximity with the ~~gene~~ coding seqeunce of interest, either upstream or downstream of the gene. An example of a CS and CS-BD may include, but are not limited to, the GAL4 binding domain (GAL4-BD) and the GAL4 upstream activating sequence (GAL4-UAS). However, it is to be understood that other recognition sequences may be used for this purpose as are known to one of skill within the art. For example, a CS may be an endogenous CS associated with a gene, that is involved within a gene expression cascade, for example but not limited to a developmental cascade. In this embodiment the CS is preferably associated with a gene that is involved at an early stage within the gene cascade, for example homeotic genes. Examples of CS and CS-DB's that are involved in initiating a gene cascade, including homeotic genes are well known to one of skill

in the art and include, but are not limited to, transcription factor proteins and associated regulatory regions, for example controlling sequences that bind AP2 domain containing transcription factors, for example, APETALA2 (a regulator of meristem identity, floral organ specification, seedcoat development and floral homeotic gene expression; Jofuku *et al.*, 1994), PRbox (pathogen resistance binding proteins), and several stress induced DNA binding proteins, or CCAAT box-binding transcription factors (e.g. *LEC*1; WO 98/37184; Lotan, T., et al.,1998, Cell 93, 1195-1205). Other examples which are not to be considered limiting in any manner of such a regulatory region include BNM3, a regulator of embryogenesis (EP 99201745.9-2105; filed June 2, 1999), or the controlling factor associated with PICKLE, a gene that produces a thickened, primary root meristem (Ogas, J., et al,.1997, Science 277, 91-94.)

Page 22, lines 30, to page 23, line 10:

The first and second regulatory elements denoted above, may be the same or different. For example, which is not to be considered limiting in any manner, the second regulatory element may be active before, during, or after the activity of the first regulatory element thereby either initially repressing expression of the ~~gene~~ coding sequence of interest followed by permitting the expression of the ~~gene~~ coding sequence of interest, or, following expression of the ~~gene~~ coding sequence of interest, the second regulatory element becomes active which results in the repression of the expression of the ~~gene~~ coding sequence of interest. Other examples, which are not to be considered limiting, include the second regulatory element being an inducible regulatory element that is activated by an external stimulus so that repression of gene expression may be controlled through the addition of an inducer. The second regulatory element may also be active during a specific developmental stage preceding, during, or following that of the activity of the first regulatory element. In this way

the expression of the ~~gene~~ coding seqeunce of interest may be repressed or activated as desired within a plant (see Examples 4 and 5).

Page 23, lines 12-18:

It is also within the scope of the present invention that the chimeric construct may comprise the elements of the expression construct, as described above, and those of the effector construct, as described above, in a contiguous manner, so that all of the elements for expressing a ~~gene~~ coding sequence of interest and expressing HD are provided for on one chimeric construct. The first and second regulatory regions may be the same or different, and selected to provide for the constitutive, developmental, temporal or induced expression of either the ~~gene~~ coding sequence of interest or HD as desired.

Page 27, line 22, to page 28, line 9:

Co-bombardment of leaves with either reporter and the control construct or reporter and GAL4 resulted in a high level of GUS activity (Figure 10 (B)), while co-bombardment with AtHD2A significantly reduced GUS activity. These results again indicate that *AtHD2A* can mediate transcriptional repression of a targeted reporter gene *in vivo*. To determine the protein domains of *AtHD2A* responsible for gene repression, a series of deletion constructs of *AtHD2A* were made (Figure 10 (B)) and tested by transient expression in Arabidopsis plants. Deletion of C-terminal residues up until the amino acid 162 of *AtHD2A* (GAL4-*AtHD2A*, 1-211 and GAL4-*AtHD2A*,1-162) did not affect the repression activity of the molecules (Figure 10 (B)). However, further deletions to the amino acid 100 of the C-terminal residue (GAL4-*AtHD2A*, 1-100) resulted in a complete loss of gene repression activity. This observation indicates that the region between the amino acid residues 101 to 161 is important for repression activity. This region also includes an extensive acidic amino acid domain, which is important for association with basic tails of histones (Philpott and Leno, 1992). Deletion of the domain containing

predicted catalytic residues (GAL4-*AtHD2A*, 73-245) resulted in complete loss of repression activity (Figure 10 (B)). Collectively, these results demonstrate that both the deacetylase catalytic activity and HD binding with histones is essential for gene repression activity. Furthermore, these results indicate that fragments or analogs of HD are active in repressing the expression of a ~~gene~~ coding sequence of interest.

Page 31, lines 4-18:

Plants transformed with UAS_{GAL4}-tCUP-GUS were crossed with either tCUP-GAL4/AtHD2A (constitutive expression) and NAP-GAL4/AtHD2A (tissue specific expression) effector lines. Analysis of the F1 progeny from a cross between UAS_{Gal4}-tCUP-GUS X 35S-GAL4/AtHD2A, and UAS_{Gal4}-tCUP-GUS X NAP1-GAL4/AtHD2A are presented in Figure 19 (A). High levels of expression of a ~~gene~~ coding sequence of interest (e.g. reporter gene activity) are observed in leaves and seeds in control plants expressing GUS under the control of the constitutive regulatory element tCUP. In F1 progeny of plants derived from a cross between UAS_{Gal4}-tCUP-GUS X 35S-GAL4/AtHD2A, reduced reporter gene expression is observed in both leaves and seeds, due to the constitutive expression of the HD/GAL4BD, and the UAS_{Gal4}-reporter genes. High levels of expression of a ~~gene~~ coding sequence of interest (e.g. a reporter gene) are observed in leaf tissue of F1 progeny derived from a cross between UAS_{Gal4}-tCUP-GUS X NAP1-GAL4/AtHD2A due to a lack of expression of the effector construct under the control of the tissue-specific promoter. However, in seed tissues, reporter expression is dramatically reduced due to the targeted expression of the HD/GAL4BD.

Page 31, line 20, to page 32, line 2:

Similar results are obtained in dual transgenic plants that have been transformed sequentially, that is, following the initial transformation of a plant with an expression gene (for example GUS), the transgenic plant is re-transform~~ation~~ed with an effector gene. As

shown in Figures 19 (B) and (C), plants transformed with both an expression construct and re-transformed with either 35S-GAL4/AtHD2A, or NAP1-GAL4/AtHD2A display similar patterns of repression of the ~~gene~~ coding sequence of interest as that observed following crossing expression X effector plant lines. In plants sequentially transformed with the reporter construct and an effector construct that is constitutively expressed in the plant (35S-GAL4/HD), repression of GUS activity is observed in both leaves and seed (-Figures 19 (B) and (C)). Repression of GUS activity is only observed in seed tissues in dual transgenic plants sequentially transformed with the expression construct followed by the seed specific effector construct NAP1-GAL4/HD. No repression of reporter gene activity was observed in leaf tissue in dual transgenic plants re-transformed with the seed specific effector construct.

Page 32, lines 13-19:

An example, which is not to be considered limiting in any manner, involves the use of an effector construct comprising HD associated with a CS-BD. The CS-BD, for example, but not limited to a transcription factor, is capable of binding an endogenous CS within the plant, thereby permitting the associated HD to repress expression of a gene associated with the CS. If the CS is associated with a gene involved with a developmental or metabolic cascade, for example but not limited to a homeotic gene, then repression of the ~~gene~~ coding sequence of interest ensures that the cascade is not initiated.

Page 33, lines 1-15:

Pti4 enhanced GCC box-mediated transcription of a ~~gene~~ coding sequence of interest with which it was co-transformed (Figure 20). Solano et al. (1998) reported that overexpression of another ERF (ethylene-responsive element binding factor) protein, ERF1, in transgenic *Arabidopsis* plants induced basic chitinase gene expression. Basic chitinase is an ethylene-responsive gene, which contains the GCC box in its promoter (Samac et al., 1990). The GCC-box contains a conserved AGCCGCC sequence, which was first identified

from the promoters of ethylene-inducible PR genes in tobacco (Ohme-Takagi and Shinshi, 1995). Without wishing to be bound by theory, it has been suggested that this sequence is a target in the ethylene signal transduction pathway because deletion of the GCC box eliminates ethylene responsiveness (Broglie et al., 1989;; Shinshi et al., 1995). Therefore, the expression of tomato *Pti4* in *Arabidopsis* was examined to determine if Pti4 could induce the expression of the *Arabidopsis* basic chitinase gene. Northern analysis also showed that expression of *Pti4* in transgenic *Arabidopsis* plants induced the expression of a GCC box-containing, endogenous, PR gene, basic chitinase, in *Arabidopsis* (Figure 21).

Page 34, lines 13-31:

Therefore the present invention is directed to a method of regulating the expression of a ~~gene~~ coding sequence of interest in a plant comprising:

i) introducing into the plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase fused with a nucleotide sequence encoding a controlling sequence binding domain that has an affinity for a native controlling sequence upstream within the ~~gene~~coding sequence, to produce a transgenic plant; and

ii) growing the transgenic plant.

The controlling sequence binding domain may be for example ~~A~~a DNA binding protein, and the controlling sequence may be an upstream activating sequence.

Similarly, the above method may be used to regulate a developmental, physiological, or biochemical pathway within a plant by introducing into the plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase fused with a nucleotide sequence encoding a DNA binding protein that has an affinity for a native upstream activating sequence within the ~~gene~~

coding sequence of interest known to be associated with a developmental, physiological or biochemical cascade, for example a homeotic gene, to produce a transgenic plant.

Page 42, lines 4-7:

Collectively, these results demonstrate that both the deacetylase catalytic activity and HD binding with histones is essential for gene repression activity. Furthermore, these results indicate that fragments or analogs of HD are active in repressing the expression of a ~~gene~~ coding sequence of interest.

Page 46, lines 11-19:

Repression of a ~~gene~~ coding sequence of interest may occur within a plant following sequential transformation of a target gene, for example GUS, followed by transformation with an effector gene. To demonstrate the efficacy of this approach, Arabidopsis plants were transformed using standard techniques (Clough and Bent,1998) using the construct UAS_{GAL4}-tCUP-GUS (tCUP-GUS; reporter gene). As shown in Figures 19 (B) and (C), these plants (indicated as control 1, 2) exhibit GUS activity in both leaves and seeds. Transformed plants expressing GUS were then re-transformed with one of two effector constructs, 35S-GAL4/AtHD2A, or NAP1-GAL4/AtHD2A. The levels of GUS activity within the dual transgenics are shown in Figures 19 (B) and (C).

Changes to Claims:

Claims 10, 20, 21, 23, 25, 27 and 28 are canceled.

Claims 29-31 are added.

The following is a marked-up version of the amended claim(s):

1. (Amended) A method of [~~regulating gene expression~~] repressing transcription of a coding sequence of interest in a transgenic plant, comprising~~:~~:

a) introducing into a plant:

_____i) a first chimeric nucleotide sequence comprising a first regulatory element in operative association with a gene of interest, and a controlling sequence; and

_____ii) a second chimeric nucleotide sequence comprising a second regulatory element in operative association with a nucleotide sequence encoding a histone [~~deaceytlase and~~] deacetylase fused with a DNA binding protein, said DNA binding protein interacting with said controlling sequence, to produce said transgenic plant; and

~~iii~~b) growing said transgenic plant.

4. (Amended) The method of claim 1, wherein said histone deacetylase, within said step of introducing, is selected from the group consisting of:

i) AtRPD3A, AtRPD3B, AtHD2A, or AtHD2B[~~,~~];

ii) [~~an analogue,~~]a fragment[~~, or derivative~~] of AtRPD3A, AtRPD3B, AtHD2A, or AtHD2B[~~,~~]; and

iii) a nucleotide sequence that hybridizes to AtRPD3A, AtRPD3B, AtHD2A, or AtHD2B at 65°C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1mM EDTA, followed by washing for 15 minutes in 2 x SSC with 0.1% SDS at room temperature, then twice for 20 minutes in 0.1 x SSC, 0.1% SDS at 65°C;

wherein said [~~analog,~~] fragment[~~, derivative,~~] or nucleotide sequence that hybridizes encodes a product that exhibits repression of gene expression activity.

9. (Amended) An isolated nucleotide sequence, selected from the group consisting of:

i) [~~SEQ ID NO:1,~~] SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7;

ii) [~~an analog, derivative,~~] a deletion or fragment of [~~SEQ ID NO:1,~~] SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7; and

iii) a nucleotide sequence that hybridizes to [~~SEQ ID NO:1,~~] SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 at 65°C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA, followed by washing for 15 minutes in 2 x SSC with 0.1% SDS at room temperature, then twice for 20 minutes in 0.1 x SSC, 0.1% SDS at 65°C;

wherein said [~~analog, derivative,~~] fragment, said deletion, or said nucleotide sequence that hybridizes encodes a product that exhibits repression of gene expression activity.

14. (Amended) A transgenic plant cell produced by the method of claim 1.

15. (Amended) A transgenic plant produced by the method of claim 1.